Polish Journal of Veterinary Sciences Vol. 21, No. 3 (2018), 549–557

DOI 10.24425/124289

Original article

Comparative analysis of cat bone marrow and adipose tissue cell cultures

A. Mazurkevych[1], M. Malyuk[1], V. Kovpak [1], O. Kovpak [1], Y. Kharkevych[1], A. Jakubczak[2], M. Gryzinska[2]

[1] Department of Physiology, Pathophysiology and Immunology of Animals, National University of Life and Environmental Sciences of Ukraine, Heroiv Oborony 15, 03041 Kyiv, Ukraine
[2] Institute of Biological Basis of Animal Production, Faculty of Biology, Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland

Abstract

Cell culture transplantation is very promising in the treatment of various diseases. Cells obtained from a number of sources have been analysed to provide a basis for further studies in the area of regenerative medicine. The objective of the study was to compare morphological and phenotypic changes in cat adipose tissue and bone marrow cell cultures from the first to fifth passages. Adipose tissue and bone marrow were used to obtain cell cultures (coming from 3 cats) using standard methods with own modification. Phenotype changes were monitored by CD-marker identification and CD pan-keratin. The cytogenetic analysis was performed on 50 metaphase plates of cell cultures from the first to fifth passage. Cytogenetic assays showed that the adipose tissue cell culture (ATCC) at all passages was more stable than the bone marrow cell culture (BMCC).

Key words: stem cells, bone marrow cell culture, adipose tissue cell culture, cytogenetic assays

Introduction

Bone marrow is well-explored today as a source of stem cells from adult donors. Bone marrow is the only tissue of the adult body that is naturally composed of immature, indifferent and poorly differentiated cells, known as stem cells (He et al. 2014, Mazurkevych et al. 2016, Mazurkevych et al. 2017). It has been demonstrated that mesenchymal and endothelial cells contained in the bone marrow can become various types of non-hematopoietic cells – osteoclasts, chondrocytes, adipocytes, and epithelial cells (Dorshkind 2002).

Adipose tissue is an alternative source of cell material that can be obtained by less invasive methods and in much larger quantities than bone marrow cells. Adipose tissue consists of adipose cells – adipocytes, as well as cells that make up the Stromal Vascular Fraction (SVF): pre-adipocytes, endothelial and smooth muscle cells of blood vessels, perivascular fibroblasts, and cells of fibrous collagenous stroma (Carswell et al. 2012).

Correspondence to: M. Gryzinska, e-mail: magdalena.gryzinska@up.lublin.pl, tel.: +48 81 445 60 68

Stem cells contained in adipose tissue are multipotent. They can differentiate into different cell lines, including adipose, bone, cartilaginous, nerve tissue, endothelial (Zuk et al. 2001, Guilak et al. 2006) and hepatic cells (Aurich et al. 2009, Ruiz et al. 2010).

Cell culture transplantation is very promising in the treatment of various diseases. Cells obtained from a number of sources have been analysed to provide a basis for further studies in the area of regenerative medicine. This study compares changes in the phenotypic traits of cells for the purpose of selecting an optimal source of cell material.

The objective of the study was to compare morphological and phenotypic changes in cat adipose tissue and bone marrow cell cultures from the first to fifth passage.

Materials and Methods

Adipose tissue and bone marrow were used to obtain cell cultures. The material was obtained during scheduled surgeries. All manipulations with animals were conducted with the prior approval of their owners and in compliance with the Law of Ukraine 'on the Protection of Animals from Cruelty' (No. 3447 – IV, dated 21.02.2006) (Law of Ukraine 2006).-

The adipose tissue cell culture (ATCC) was obtained from subcutaneous tissue using standard methods (Freshney 2005, Bunnell et al. 2008, Carswell et al. 2012) with our own modification. The bone marrow cell culture (BMCC) was obtained mainly from femoral bone marrow using standard methods (Freshney 2005). The cell mass was cultured in standard conditions: 80% DMEM; 20% FBS; 10 mcL/cm^3 antibiotic-antimycotic (Sigma, USA); in a CO_2 incubator at 37°C and a 5% CO_2 concentration (Freshney 2005), to a confluency of 90-100%. Further passaging was performed at a 1:3 dilution. Microscopic analysis and culture assessment were performed with an Axiovert 40 inverted microscope (Carl Zeiss).

Phenotype changes were monitored by CD-marker identification (CD10, CD38, CD34, CD45, CD48, CD54, CD56, CD66e, CD96, CD227, CD3266, and CD pan-keratin). Specimens were prepared using standard methods (Freshney 2005). The results were analysed according to the number of cells with antigen expression (green glow of cells) and were assessed by the classical H-Score method: $S=1\times A + 2\times B + 3\times C$, where S – H-Score index, with a value between 0 (protein is not expressed) and 300 (strong expression of protein in 100% of cells); A – percentage of cells with weak expression of the protein; B – percentage of cells with moderate expression; C – percentage of cells with strong expression (Igarashi et al. 2016, Mazurkevych et al. 2016, Mazurkevych et al. 2017). The level of expression was defined as negative if the score ranged from 0 to 50; low – from 51 to 100; moderate – from 101 to 200; and high – 201 and higher. The cells were examined with a Leica DMR fluorescent microscope (Germany).

Additionally, a cytogenetic analysis of the cultures was performed. The analysis was performed on 50 metaphase plates of cell cultures from the first to fifth passage. A modification of a standard cytogenetic method was used to obtain slides (Freshney 2005). The slides were stained using a staining kit (Leikodif 200) according to the manufacturer's instructions. Metaphase plates were analysed with a Leica DMR microscope (Germany) at ×400 and ×1000 magnification.

Results

Comparative analysis of the morphology of adipose tissue and bone marrow cell cultures

Primary adherent cell cultures of cat adipose tissue and bone marrow were morphologically heterogeneous.

Within several days after inoculation a significant number of low-adhesive flat-angulated cells were observed, which were removed during the passaging process. From day 3 for BMCC and day 5 for ATCC, growth of fibroblast-like cells was noted. Primary ATCC reached 90-100% confluency in 12 days on average (Fig. 1a) and BMCC in 8 days (Fig. 1b).

In the subcultivation process, the time to reach 70-80% confluency was 4 days for ATCC at passages 1-3 and increased to 6 days at passages 4-5. The corresponding time for BMCC was 3 days on average during all 5 passages. At the first passage, heterogeneity of cultures was observed for both bone marrow and adipose tissue, including a small number of polygonal-like cells and fibroblast-like cells. The number of polygonal-like cells decreased in both ATCC and BMCC with every passage. The most homogeneous composition of cultures was observed at the fifth passage, with morphology characterised mainly by a fibroblast-like structure (Figs. 2a, 2b).

Characteristics of adipose tissue and bone marrow cell cultures using surface markers

The analysis of bone marrow and adipose tissue cell cultures showed differences in their immunophenotype that did not disappear with passages. The changes in CD markers examined in the samples are presented in

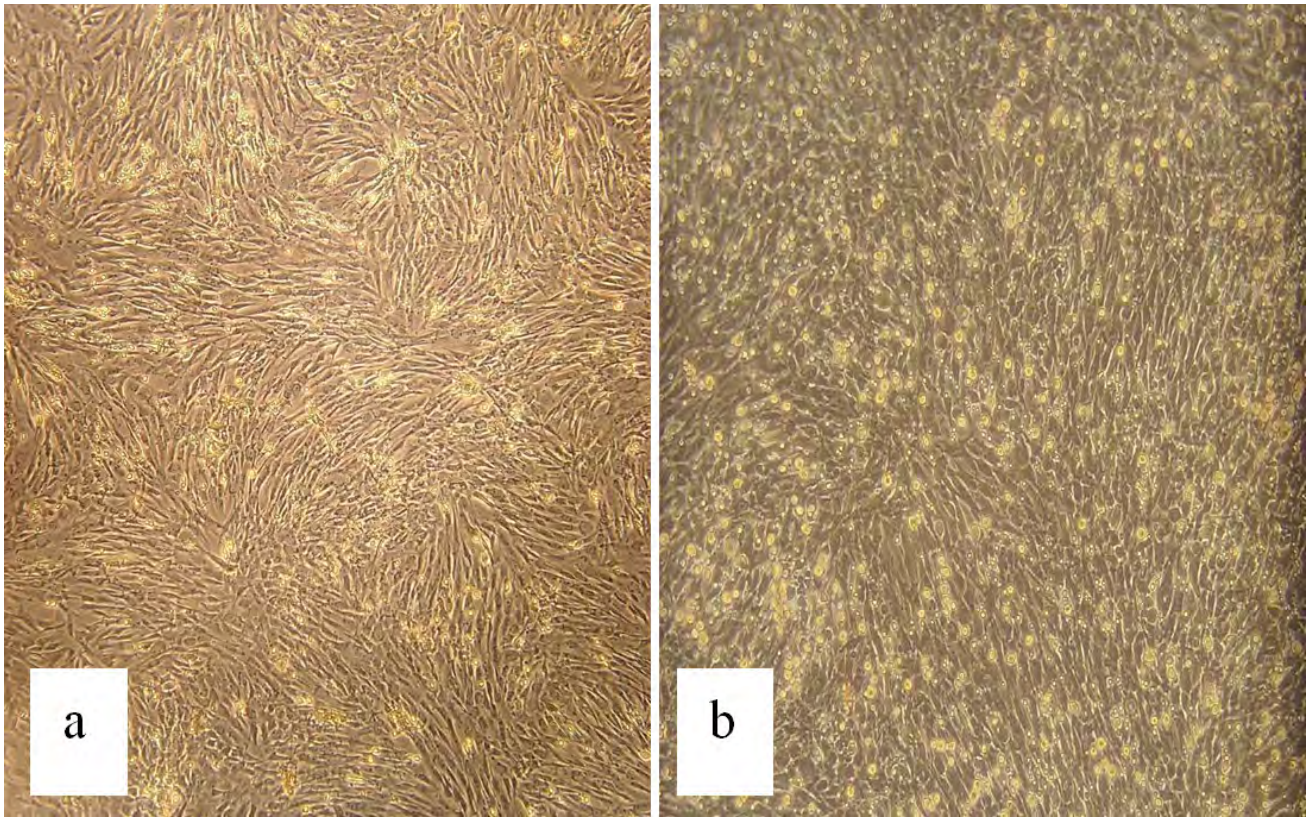

Fig.1. Photomicrography of the cell culture in vitro: a - adipose tissue, 12th day of cultivation (0 passage); b - bone marrow, 8th day of cultivation (0 passage). Negative slide. Magnification ×10, sp ×5.

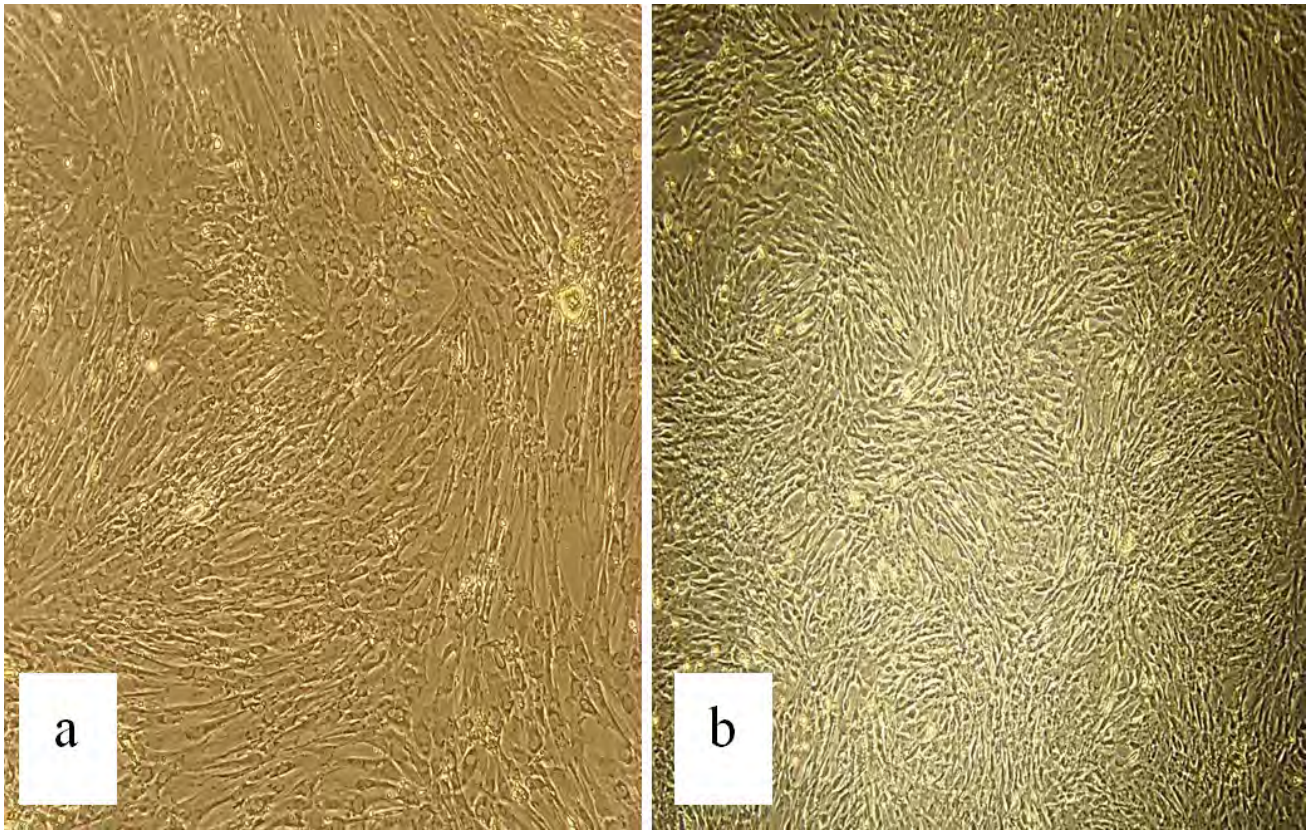

Fig. 2. Photomicrography of the cell culture in vitro: a - adipose tissue, 5th passage; b - bone marrow, 5th passage. Negative slide. Magn. ×10, sp ×5.

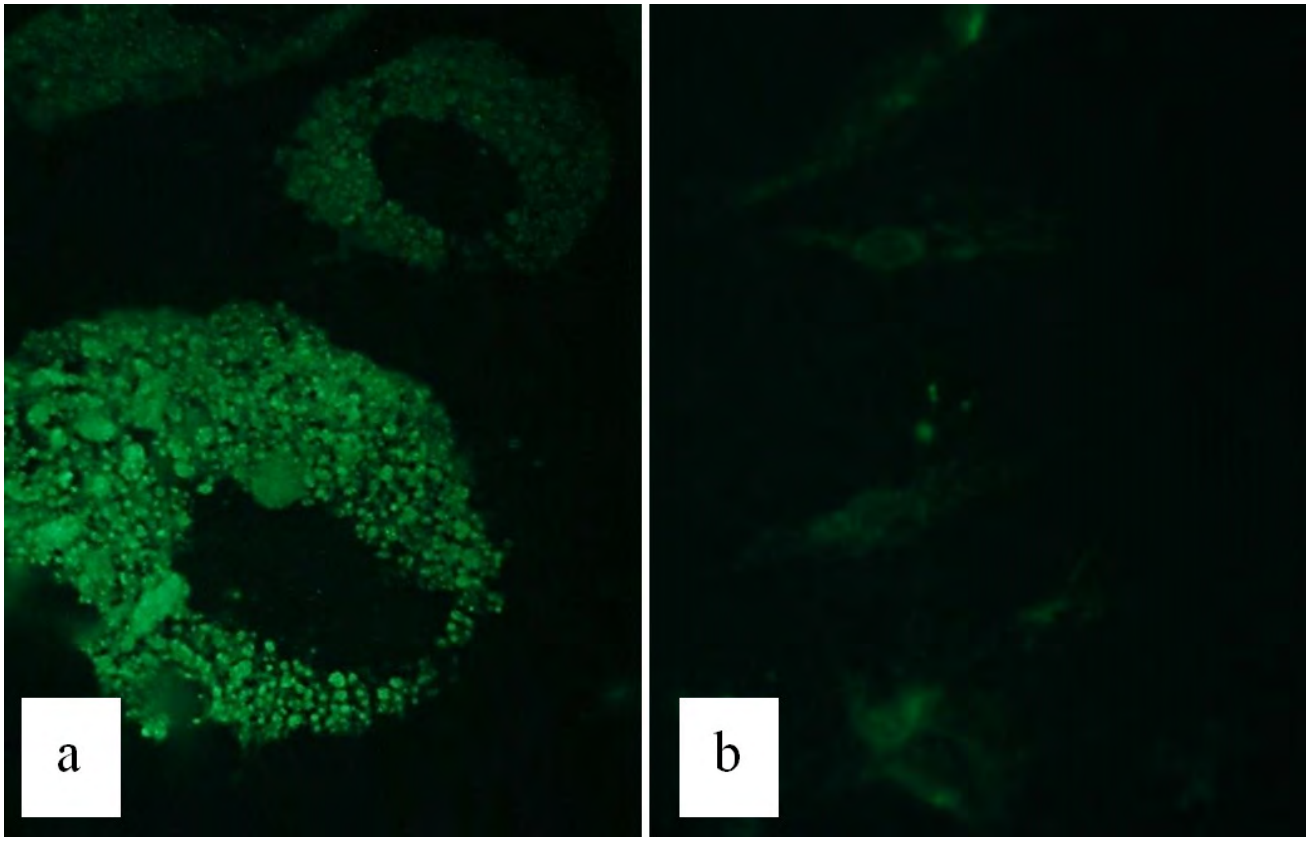

Fig. 3. Photomicrography of CD54 expression level in the cell culture of cat: a- adipose tissue, 4th passage; b - bone marrow, 4th passage. Fluorescence microscopy. Magn. ×10, sp ×100.

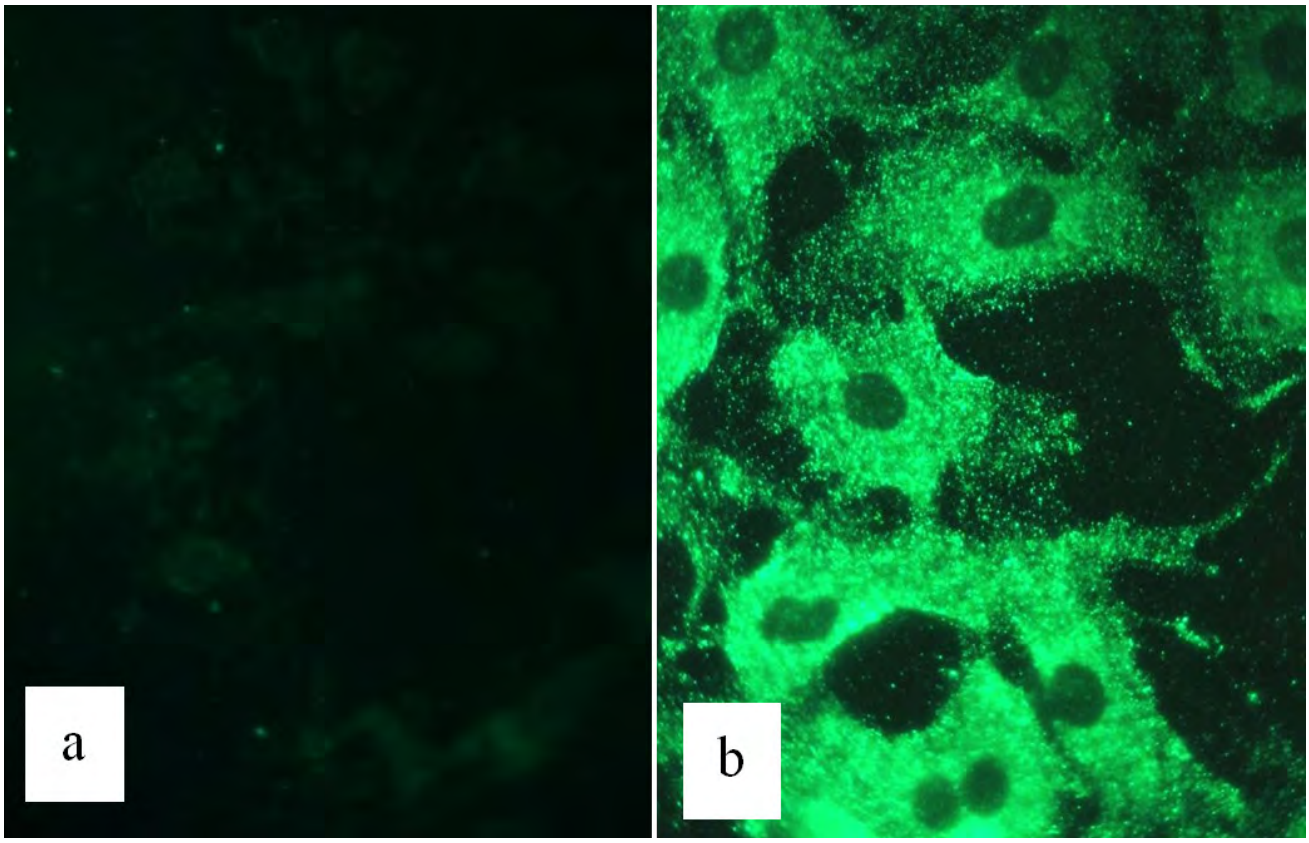

Fig. 4. Photomicrography of pan-keratin expression level in the cell culture of cat: a - adipose tissue, 1st passage; b - bone marrow, 1st passage. Fluorescence microscopy. Magn. ×10, sp×100.

Table 1. Comparison of changes in CD marker expression in the adipose-derived and bone-marrow-derived cell population of cats from first to fifth passage (M±m, n=3).

CD markers	Cell culture	Passage				
		1	2	3	4	5
		Scores according to the H-Score method (from 0 to 300)				
10	BMCC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ATCC	0.0 ± 0.0	16.7 ± 5.4*	18.0 ± 5.2*	94.3 ± 8.3***	62.3 ± 6.2***
34	BMCC	111.7 ± 7.9	105.3 ± 6.0	94.0 ± 5.8	91.7 ± 6.8	90.0 ± 5.8
	ATCC	148 ± 10.8	131.0 ± 18.0	86.3 ± 13.0*	55.7 ± 7.7**	31.7 ± 7.9**
38	BMCC	110.7 ± 6.2	92.7 ±5.0	61.7 ± 10.6 *	46.0 ± 9.2**	23.3 ± 6.1***
	ATCC	9.3 ± 5.4	43.0 ± 6.4*	49.7 ± 2.5**	70.7 ± 7.2**	73.7 ± 6.6**
45	BMCC	92.7 ± 4.5	83.0 ± 4.1	75.0 ± 3.5*	65.3 ± 3.7**	52.7 ± 9.7*
	ATCC	13.7 ± 2.7	16.7 ± 3.7	14.7 ± 2.7	16 ± 2.3	76.3 ± 11.8**
48	BMCC	60.1 ± 7.7	68.7 ± 6.6	77.3 ± 5.4	92.3 ± 5.4*	106.7 ± 9.4*
	ATCC	96.0 ± 15.7	59.3 ± 11.4	27.3 ± 5.4*	7.3 ± 4.3**	139.0 ± 6.4
54	BMCC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.3 ± 3.7	7.0 ± 4.1
	ATCC	0.0 ± 0.0	0.0 ± 0.0	44.3 ± 8.3**	62.7 ± 10.6**	36.7 ± 4.8**
56	BMCC	68.3 ± 7.9	71.3 ± 10.3	77.0 ± 9.2	84.3 ± 6.8	101.7 ± 7.7*
	ATCC	0.0 ± 0.0	0.0 ± 0.0	28.3 ± 9.7*	45.7 ± 7.7**	44.0 ± 6.4**
66e	BMCC	58.0 ± 10.5	69.0 ± 9.2	91.3 ± 2.5*	98.3 ± 8.9*	113.0 ± 11.0
	ATCC	0.0 ± 0.0	7.0 ± 4.1	20.0 ± 4.1**	61.3 ± 6.0***	72.0 ± 7.0***
95	BMCC	52.7 ± 4.5	71.3 ± 7.7	86.0 ± 5.2**	108.0 ± 11.0**	107.3 ± 14.3*
	ATCC	102.7 ± 12.39	145.3 ±11.8	26.3 ± 7.4**	53.3 ±14.1	67.3 ±16.1
227	BMCC	55.0 ± 4.6	64.3 ± 5.6	81.7 ± 2.5**	88.0 ± 1.7**	92.7 ± 4.5**
	ATCC	0.0 ± 0.0	6.3 ± 3.7	21.0 ± 7.0*	34.0 ± 8.7*	65.7 ± 5.6***
326	BMCC	92.7 ± 4.5	71.0 ± 12.8	52.7 ± 2.1*	34.7 ± 6.0**	25.0 ± 8.1**
	ATCC	76.0 ± 5.2	65.7 ± 6.8	15.3 ± 5.6**	26.7 ± 4.5 **	70.0 ± 8.1
pan-keratin	BMCC	234.3 ± 15.5	226.7 ± 13.6	216.3 ± 15.5	188.3 ±6.8	172.3 ± 11.8*
	ATCC	0.0 ± 0.0	19.0 ± 5.8*	28.3 ± 3.9**	60.7 ± 6.8***	84.3 ± 6.0***

* p<0.05; ** p< 0.01; *** p< 0.001 compared to control (first passage was control for every CD marker)

Figs. 3a and 3b and in the Table 1., while Figs. 4a and 4b show pan-keratin expression level in the cell culture.

Cytogenetic assay of the bone marrow and adipose tissue cell cultures

The karyotype test of cat bone marrow and adipose tissue cell cultures showed that they had quantitative abnormalities (aneuploidy and polyploidy) (normal cat karyotype n=38 (Fig. 5a). The results are shown in Table 2.

Chromosomal plates with an aneuploidy were observed from the first to sixth passage in both cultures. Their lowest percentage, both in the bone marrow cell culture (9.3%) and in the adipose tissue culture (6.7%), was observed in the first passage. The most cells with numerical chromosome aberrations were noted at the fifth passage (BMCC – 17.3%, ATCC – 16.0%). These abnormalities can be explained by the fact that during cultivation the number of cells with an abnormal chromosome set increases through their uncontrolled division. It is worth noting that there were fewer cells with aneuploidy in the adipose tissue cell culture than in the bone marrow cell culture, which demonstrates its greater genetic stability.

Metaphase plates with different numbers of chromosomes (Fig. 5b) were detected in the bone marrow cell culture from the first (2.7%) to the fifth (5.3%) passage and in the adipose tissue from the first (5.3%) to the fourth passage (0.7%) (Table 2).

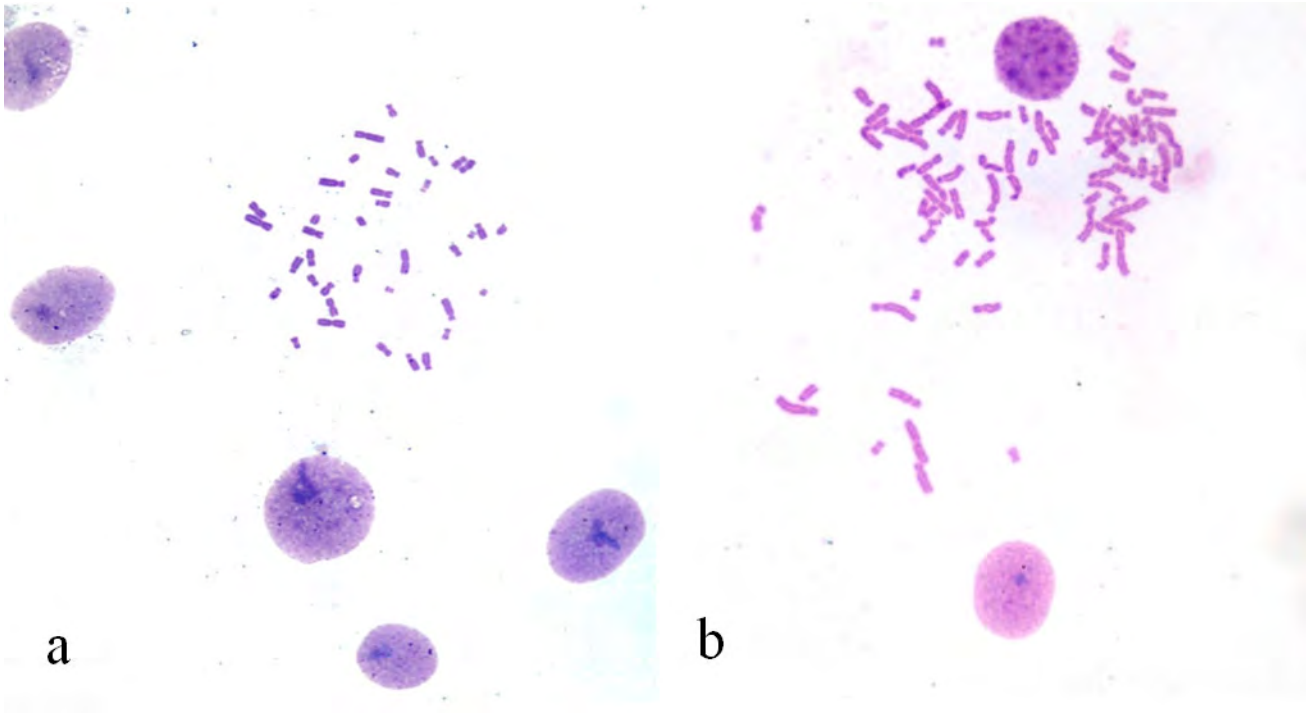

Fig. 5. Photomicrography of cat metaphase plate: a - normal karyotype, n=38; b - polyploidy, 2n=76. Leikodif 200 staining. Magn. ×10, sp ×100.

Table 2. Cytogenetic assay results of cat bone marrow and adipose tissue cell cultures, passages 1-5, (M±m, n=3).

Passage No.	Aneuploidy, %		Polyploidy, %	
	BMCC	ATCC	BMCC	ATCC
I	9.3 ± 0.8	6.7 ± 0.8	2.7 ± 0.8	5.3 ± 0.8
II	11.3 ± 1.5	7.3 ± 0.8	3.3 ± 0.8	2.0 ± 1.2
III	16.7 ± 0.8**	10.7 ± 1.5	4.0 ± 0.0	0.7 ± 0.8*
IV	16.0 ± 0.0**	14.0 ± 1.2**	5.3 ± 0.8	0.7 ± 0.8*
V	17.3 ± 0.8**	16.0 ± 1.2 **	5.3 ± 0.8	0.0 ± 0.0**

* p<0.05; ** p<0.01; *** p<0.001, compared to control (first passage was control)

Discussion

During passaging, the CD profile in the cell cultures changed in definable ways. CD10, or neutral endopeptidase, belongs to the metalloproteinase family (Shipp et al. 1989). Its level of expression was defined as negative during the entire passaging period in the bone marrow cell culture, and as low at the 4th and 5th passages in the adipose tissue cell culture (Table 1). CD34 is a type I transmembrane monomeric glycoprotein that mediates intercellular adhesion. It is a marker of hematopoietic stem cells, endothelial vascular cells, and embryonic fibroblasts (Krause et al. 1996). During the study, a slight decrease in its expression with passages was observed in the bone marrow cell culture, while in ATCC the intensity of CD34 decreased sharply and was negative at the fifth passage. CD38 is a type II single-strand transmembrane glycoprotein (Alessio et al. 1990) whose molecule is an intermediate of several different activities, including signal transduction, cell adhesion and cyclic ADP-ribose synthesis (Malavasi et al. 1994). The highest level of CD38 expression, which decreased with passages, was observed at the first passage in the bone marrow cell culture. This may indicate that the level of cell differentiation in the culture is lowest at the beginning of passaging and increases with the duration of cultivation. The reverse pattern was observed in ATCC. CD45 is a type I transmembrane glycoprotein that belongs to the protein-tyrosine phosphatase family. It is expressed in all hematopoietic cells except for red blood cells and platelets (Trowbridge and Thomas 1994). This may explain the steady

decrease in the expression of this marker in the BMCC from 92.7 (1st passage) to 52.7 (5th passage) points. A small number of cells expressing CD45 was observed at the first passages of the adipose tissue cell cultures, but their number increased sharply at the fifth passage. Expression of CD45 is typical for adipose tissue (Astori et al. 2007). CD48 is a type I transmembrane glycoprotein bound to the cell membrane through phosphatidylinositol glycoside (Shin and Abraham 2001). CD48 is expressed in some hematopoietic and endothelial cells. It participates in the activation and programmed differentiation of these cells. During the study, a steady decrease in expression, from low to negative, was observed in ATCC, with a sharp increase at the fifth passage. The level of CD48 expression increased steadily from low to moderate in BMCC (Table 1).

CD54 is a type I single-strand transmembrane glycoprotein that is present on endothelial cell membranes and is important for leukocyte adhesion (Gay et al. 2011). During the entire cultivation period no CD54, was observed in BMCC (Fig. 3a), while a steady increase in its expression was observed in ATCC at the third (44.3 points) and fourth (62.7 points) (Fig. 3b) passage, with a slight decrease at the fifth passage (36.7 points) (Table 1). CD56 is a type I transmembrane glycoprotein. It is an isoform of a neural cell adhesion molecule that mediates neurogenesis and a marker of NK cells and plasma cells (Cunningham et al. 1987). During the first two passages its expression was not observed in ADCC; although its level increased steadily from the third passage, it never exceeded the negative range. In BMCC, a gradual increase in the expression of this marker was observed, peaking at the fifth passage (68.3 points – 1st passage; 101.7 – 5th passage). CD66e is a glycated glycoprotein of the epithelial cell surface, which explains its detection in most organs (Hammarström 1999). Its expression increased steadily from the first (no expression) to fifth passages (low level) in the adipose tissue cell culture. A similar pattern was observed in BMCC, from a low level at the first passage (58.0 points) to a moderate level at the fifth passage (113.0 points). CD95 is a type I transmembrane glycoprotein that mediates the apoptosis-inducing signal (Yonehara et al. 1989). Starting from the first passage, the intensity of expression of this marker increased steadily in BMCC, which was correlated with a decline in culture growth. The level of CD95 expression in ATCC was irregular, which can be explained by the irregularity of the culture growth. CD227 is a transmembrane glycoprotein expressed by epithelial and some hematopoietic cells (Inagaki et al. 2009). Hyperexpression of this marker leads to transformation of cells and blocks a stress-induced apoptosis through Akt or p53 cascades (Raina et al. 2004). During the study we observed a low level of CD227 expression. Its level increased steadily from 55.0 points at the first passage to 92.7 at the fifth in BMCC. A lower level of expression was observed in ATCC, increasing from negative at the first passage to low (65.7 points) at the fifth. CD326 is a type I transmembrane glycoprotein which is a marker of epithelial cells. Cells that express this marker have reduced demand for growth factor and increased metabolic activity and colony-forming ability (Münz et al. 2004). A decrease in CD326 expression from low to negative was observed in the bone marrow cell culture. In ATCC, a decrease in expression of this marker was observed from the first (76.0 points) to the third passage (15.3 points), with incremental recovery of its initial expression at the fifth passage (70.0 points) (Table 1).

Keratin is a component of intermediate filaments of the cytoskeleton of epithelial cells (Chang and Goldman 2004). It should be noted that cytokeratin has been detected in adipose tissue by other scientists (Yang et al. 2015). In our study, the level of keratin expression in the adipose tissue cell culture increased from 0 (1st passage) (Fig. 4a) to 84.3 points (5th passage). A steady decrease in this protein from high to moderate was observed in the bone marrow cell culture (Fig. 4b) (Table 1).

The final mechanism of polyploid development is as yet unknown. There are, however, a few hypotheses: deviation from the normal mitosis process as a result of fusion of two cells (more common for cell cultures), or mitotic deceleration or acceleration (Matsyara 1980). Observations of patterns of appearance of polyploidy in the cultures under study indicate that the mechanism of their appearance is different in the BMCC and ATCC. The presence of polyploidy in ATCC is determined by rapid cell division, while in BMCC it is caused by cell fusion during long-term cultivation and by deviation from the normal mitosis process (Andraszek et al. 2016, Chomik et al. 2016).

Conclusions

The results of our study have shown that the cultures of initial adherent cells of both adipose tissue and bone marrow consist of different types of cells. The percentage of fibroblast-like cells increased in the cell cultures with successive passages.

On the basis of the spectrum of CD-markers used, which is typical for nonspecialized (poorly differentiated) cells, the lower level of expression in ATCC (compared to BMCC) may indicate a higher level of universality for the bone-marrow-derived cell culture. This hypothesis is confirmed by the deceleration of single

layer formation in cell cultures starting from the fourth passage, while in the BMCC the time required to reach 90-100% confluency was stable.

The cytogenetic assays showed that the ATCC was more stable at all passages than the BMCC. These values did not exceed the limits of typical natural mutation of mammalian somatic cells in terms of numerical chromosome aberrations, which indicates the genetic stability of the cell cultures under study.

The occurrence of different phenotypes in the bone marrow and adipose tissue cell cultures will make it possible to use them in cell-based therapies in the future, and they can be expected to have varying impact under the same conditions.

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